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Malonylated anthocyanidin 3,5-diglucosides in the flowers of the genus *Disa* (Orchidaceae)

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Keywords: *Disa* cultivars; Orchidaceae; cyanidin 3,5-diglucoside; pelargonidin 3,5-diglucoside; malonic acid

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1. Subject and Source

Recently we have detected the occurrence of pelargonidin and cyanidin in the flowers of *Disa* hybrids (Tatsuzawa et al., 2010a). In the present study, we further investigated the detailed structures of anthocyanins in the red-purple, red, and orange-red flowers of *Disa* cultivars, grown by Hokkaisankyo Co. Ltd (Hokkaido, Japan), and identified them as acylated and non-acylated pelargonidin and cyanidin 3,5-diglucosides. The distribution of these anthocyanins in Orchidaceae was discussed along with the classification by phylogenetic analysis of Orchidaceae.

Voucher specimens are deposited at National Museum of Nature and Science (TNS).

2. Previous work

There are only two previous reports on flavonoids from the genus *Disa*. Flavon *C*-glycosides were detected in leaf material of *Disa uniflora* Berg. (Williams, 1979). More recently, we have reported the distribution of cyanidin and pelargonidin as the aglycones of anthocyanins in the flowers of cultivars of the given genus (Tatsuzawa et al., 2010a).

3. Present study

3.1. Isolation and identification of anthocyanins

By the analysis of HPLC [HPLC was performed on a LC 10A system

(Shimadzu), using a Waters C18 (4.6 ϕ x 250 mm) column at 40°C with a flow rate of 1 ml/min, the eluate was monitored at 530 nm. The eluant was applied to a linear gradient elution for 40 min from 20 to 85 % solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O)], more than 20 anthocyanin peaks were observed in the extract from the flowers of red cultivar *Disa* Transvaal 'Dawn Angel' (Figure 1). Anthocyanins **1** – **3** were easily identified to be cyanidin 3,5-di-glucoside, pelargonidin 3,5-di-glucoside and cyanidin 3-(6-malonyl)-glucoside-5-glucoside (Figure 2) with authentic samples obtained from the pink and purple flowers of *Centaurea cyanus* (Takeda et al., 1988; Goto and Kondo, 1991) by co-TLC, co-HPLC and UV-VIS spectrometry (Tatsuzawa and Shinoda, 2005) (See in Section 3.1.1. – 3.1.3.). Pigment **5** was identified by the analysis of FAB-MS and ^{1}H NMR measurement (Section 3.2.). Pigment **4** was identified by the analysis of partial acid hydrolysis of pigment **5** and FAB-MS (Section 3.3.). Moreover, pigment **6** was identified by the analysis of FAB-MS (Section 3.4.).

Dried corolla mixture of *Disa* red cultivars (60 g) were immersed in 5% HOAc-MeOH (acetic acid-methanol, 5:95, v/v, 500 ml), kept at 4°C for 1 h and extracted. The extract was concentrated to 50 ml. Anthocyanin pigments in the concentrated extract were purified by prep. HPLC [HPLC was performed on a LC 10A system (Shimadzu), using a Waters C18 (19 ϕ x 150 mm) column at 40°C with a flow rate of 1 ml/min, the eluate was monitored at 530 nm.

The eluant was applied to a linear gradient elution for 40 min from 20 to 85 % solvent B in solvent A] after thin layer and paper chromatography (BAW: BuOH-HOAc-H_2O, 4:1:2, v/v/v and 15% HOAc). Finally, pigments **1** (*ca.* 0.5 mg), **2** (*ca.* 0.5 mg), **3** (*ca.* 0.5 mg), **4** (*ca.* 3 mg), **5** (*ca.* 5 mg) and **6** (*ca.* 2 mg) were obtained as the major anthocyanins.

On hydrolysis of pigments **4** and **5** with 2N HCl for 3 days at 25°C, cyanidin 3,5-diglucoside was obtained in its hydrolysate. Similar hydrolysis of pigment **6** afforded pelargonidin 3,5-diglucoside. Moreover, malonic acid was detected in both of the hydrolysates.

3.1.1. ***Cyanidin 3,5-diglucoside (1)***: UV-VIS in 0.1% HCl-MeOH; λ_{max} 526,270 nm, E_{440}/E_{max}(%)=16, $AlCl_3$ shift +, TLC; R_f-values BAW (*n*-BuOH-HOAc-H_2O, 4:1:2, v/v/v) 0.02, BuHCl (*n*-BuOH-2N HCl, 1:1, v/v, upper phase) 0.01, 1%HCl 0.05, AHW (HOAc-HCl-H_2O, 15:3:82, v/v/v) 0.19, HPLC; t_R(min) 13.0.

3.1.2 ***Pelargonidin 3,5-diglucoside (2)***: UV-VIS in 0.1% HCl-MeOH; λ_{max} 507,267 nm, E_{440}/E_{max}(%)=21, $AlCl_3$ shift 0, TLC; R_f-values BAW 0.07, BuHCl 0.04, 1%HCl 0.13, AHW 0.35, HPLC; t_R(min) 14.9.

3.1.3. ***Cyanidin 3-(6-malonyl)-glucoside-5-glucoside (3)***: UV-VIS in 0.1% HCl-MeOH; λ_{max} 526,278 nm, E_{440}/E_{max}(%)=16, $AlCl_3$ shift +, TLC;

R_f-values BAW 0.08, BuHCl 0.08, 1%HCl 0.04, AHW 0.12, HPLC; t_R(min) 16.6.

3.2. Pigment 5

The molecular ion $[M]^+$ of pigment **5** was observed at 783 *m/z* by the FAB-mass analysis, indicating the presence of one molecule of cyanidin and two molecules each of malonic acid and glucose. The FAB-MS ion fragmentation was observed as follows, at *m/z* 697 $[M\text{-}86]^+$ loss of malonic acid; *m/z* 611 $[M\text{-}86\text{-}86]^+$ loss of two malonic acids; *m/z* 535 $[M\text{-}86\text{-}162]^+$ loss of malonic acid and glucose; *m/z* 449 $[M\text{-}86\text{-}86\text{-}162]^+$ loss of two malonic acids and one glucose; *m/z* 287 $[M\text{-}86\text{-}86\text{-}162\text{-}162]^+$ loss of two malonic acid and two glucose (=aglycone of cyanidin) supporting that the two malonic acids were linked on cyanidin 3,5-diglucoside. Therefore, the pigment was identified as dimalonyl cyanidin 3,5-diglucoside. The structure of pigment **5** was further elucidated by investigation of its ^{1}H NMR spectra [500 MHz for ^{1}H spectra in TFA-DMSO-d_6 (1:9)], including 2D COSY and negative difference NOE (DIFNOE) spectra. The ^{1}H NMR spectrum of **5** showed the presence of one molecule of cyanidin, two molecules each of glucose and malonic acid (see section 3.2.1.). These proton signals were mainly assigned by ^{1}H-^{1}H COSY, and linkages between cyanidin and sugars were confirmed by DIFNOE spectra. The proton signals of the sugar parts were observed in the region of δ 5.54 – 3.21, and two anomeric protons were exhibited at δ 5.54

112 (*d*, *J*=8.0 Hz, Glc A) and δ 5.21 (*d*, *J*=7.7 Hz, Glc B). The assigned sugar
113 protons having the coupling constants at *J*=7.7 – 12.2 Hz indicated both
114 glucose units must be β-glucopyranose. Four methylene protons were
115 assigned to H-6a and 6b of Glc A (δ4.23 and 4.40) and those of Glc B (δ4.14
116 and 4.48) by the DIFNOE experiments and also were correlated to each
117 anomeric protons by analysis of the ^{1}H-^{1}H COSY spectrum. This result
118 indicated that these two glucose units were acylated at the OH-6 groups with
119 acids, respectively. Thus, malonic acids were attached to the OH-6 groups of
120 Glc A and B, respectively.
121 In order to determined the linkages and position of the glucose units
122 DIFNOE spectra of **5** were measured. Observed NOEs between H-1 of Glc A
123 and H-4 of cyanidin indicates that Glc A is attached to the OH-3 of cyanidin
124 through a glucosidic bond. Glc B was determined to be attached to the OH-5
125 of cyanidin through a glucosidic bond, because of the presence of NOEs
126 between H-6 of cyanidin and H-1 of Blc B. Therefore, **5** is determined to be
127 cyanidin 3,5-di-*O*-[6-*O*-(malonyl)-β-glucopyranoside] (Figure 2). This is the
128 first report on the presence of cyanidin 3,5-dimalonylglucoside in the
129 Orchidaceae, although this pigment has been found in Lamiaceae plants
130 (Saito and Harborne, 1992) and *Dahlia variabilis*, belonging to Compositae
131 (Takeda et al., 1986).
132
133 **3.2.1.** ***Cyanidin 3,5-di-O-[6-O-(malonyl)-β-glucopyranoside] (5)***: UV-VIS

in 0.1% HCl-MeOH; λ_{max} 527,278 nm, E_{440}/E_{max}(%)=15, $AlCl_3$ shift +, TLC; R_f-values BAW 0.05, BuHCl 0.07, 1%HCl 0.15, AHW 0.41, HPLC; t_R(min) 20.5, ^{1}H NMR; δ cyanidin: 8.47 (s, H-4), 6.92 (d, J=2.0 Hz, H-6), 6.95 (d, J=2.0 Hz, H-8), 8.04 (d, J=2.4 Hz, H-2'), 7.08 (d, J=8.6 Hz, H-5'), 8.27 (dd, J=2.4, 8.6 Hz, H-6'). Glucose A: 5.54 (d, J=8.0 Hz, H-1), 3.62 (t, J=8.4 Hz, H-2), 3.45 (m, H-3), 3.28 (m, H-4), 3.95 (m, H-5), 4.23 (m, H-6a), 4.40 (brd, J=12.2 Hz, H-6b). Glucose B: 5.21 (d, J=7.7 Hz, H-1), 3.51 (m, H-2), 3.21 (m, H-3), 3.25 (m, H-4), 3.82 (m, H-5), 4.14 (dd, J=7.7, 11.9 Hz, H-6a), 4.48 (m, H-6b). Malonic acid (attached to OH-6 of Glc A): -CH_2-: 3.44 (s). Malonic acid (attached to OH-6 of Glc B): -CH_2-: 3.43 (s).

3.3. Pigment 4

The molecular ion $[M]^+$ of pigment **4** was observed at 697 *m/z* by the FAB-mass analysis indicating the presence of one molecule of cyanidin and malonic acid, and two molecules of glucose. The FAB-MS fragmentation at 611 *m/z* $[M-86]^+$ loss of malonic acid, at 449 *m/z* $[M-86-162]^+$ loss of malonic acid and glucose, and at 287 *m/z* aglycone indicated that the malonic acid was linked one of the glucoses of cyanidin 3,5-diglucoside. In order to obtain the authentic anthocyanin, cyanidin 3-glucoside-5-(6-malonylglucoside, the partial acid hydrolysis of pigment **5** was performed by the procedure described previously (Saito et al., 2008) providing cyanidin 3,5-diglucoside (= pigment **1**), cyanidin 3-(6-malonylglucoside)-5-glucoside (= pigment **3**), and

cyanidin 3-glucoside-5-(6-malonylglucoside) as the major anthocyanin products from the hydrolysate. The structures of these pigments were confirmed by the analysis of TLC, HPLC and FAB mass spectra. By direct comparison of pigment **4** with one of the partial hydrolysate of pigment **5**, cyanidin 3-glucoside-5-(6-malonylglucoside), both pigments were identical to by the analysis of TLC, HPLC, and the properties of UV and Vis. Therefore, pigment **4** is cyanidin 3-*O*-glucoside-5-*O*-(6-*O*-malonyl)-glucoside (Figure 2), which is a new anthocyanin in plant (Andersen and Jordheim, 2006; Harborne and Baxter, 1999; Honda and Saito, 2002).

3.3.1. *Cyanidin 3-glucoside-5-(6-malonyl)-glucoside (4)*: UV-VIS in 0.1% HCl-MeOH; λ_{max} 526,278 nm, E_{440}/E_{max}(%)=16, $AlCl_3$ shift +, TLC; R_f-values BAW 0.06, BuHCl 0.06, 1%HCl 0.05, AHW 0.16, HPLC; t_R(min) 18.0.

3.4. Pigment 6 and non purified pigments A and B

The molecular ion $[M]^+$ of pigment **6** was observed at 767 *m/z* by the FAB-mass analysis indicating the presence of one molecule of pelargonidin and two molecules each of malonic acid and glucose. The FAB-MS flagmentations at 681 *m/z* $[M-86]^+$ loss of malonic acid, at 519 *m/z* $[M-86-162]^+$ loss of malonic acid and glucose, at 271 *m/z* aglycone suggesting that the two malonic acids were linked on pelargonidin 3,5-diglucoside.

178 Therefore, the pigment **6** was identified as dimalonyl pelargonidin
179 3,5-diglucoside. By the partial hydrolysis of pigment **6** with 2N HCl for 24 h
180 at 25°C, pelargonidin 3,5-diglucoside (pigment **2**), pigment **A** [t_R (min) 18.7]
181 and pigment **B** [t_R (min) 19.9] were detected by the analysis of HPLC.
182 Therefore, pigments **2**, **A**, **B** and **6** were deduced as the pelargonidin
183 derivatives as for the cyanidin derivatives of pigments **1**, **3**, **4** and **5**,
184 respectively. Further structure elucidation of these pigments could not be
185 carried out because of small amounts available. Therefore, these three
186 anthocyanins were tentatively determined to be pelargonidin
187 3-(6-malonyl)-glucoside-5-glucoside as pigment **A**, pelargonidin
188 3-glucoside-5-(6-malonyl)-glucoside as pigment **B** and pelargonidin
189 3,5-di-(6-malonyl)-glucoside as pigment **6**, respectively, at present.
190
191 **3.4.1.** ***Dimalonyl pelargonidin 3,5-diglucoside (6)***: UV-VIS in 0.1%
192 HCl-MeOH; λ_{max} 510,268 nm, E_{440}/E_{max}(%)=20, $AlCl_3$ shift 0, TLC;
193 R_f-values BAW 0.15, BuHCl 0.14, 1%HCl 0.24, AHW 0.54, HPLC;
194 t_R(min) 22.3.
195
196 ***3.5. Distribution of anthocyanins***
197 Dried *Disa* flowers of ca. 10 mg each in dry weight of five cultivars were
198 immersed in MAW (MeOH-HOAc-H_2O, 4:1:5, v/v/v, 1ml) and extracted.
199 Analytical HPLC was performed on a LC 10A system (Shimadzu), using a

200 Waters C18 (4.6 ϕ x 250 mm) column at 40°C with a flow rate of 1 ml/min, the
201 eluate was monitored at 530 nm. The eluant was applied to a linear gradient
202 elution for 40 min from 20 to 85 % solvent B in solvent A. The results of
203 HPLC measurement at 530 nm are as follows:
204
205 **3.5.1. *Disa* Child Safety Transvaal 'Dawn Angel'**: **1** (6.3%), **2** (2.6%), **3**
206 (9.0%), **4** (31.6%), **5**(37.8%), **6** (2.4%), A (1.1%) and B (3.3%).
207 **3.5.2. *D.* Foam 'San Francisco'**: **1** (4.4%), **2** (1.4%), **3** (7.1%), **4** (29.2%),
208 **5**(38.3%), **6** 9.8%), A (2.2%) and B (5.2%).
209 **3.5.3. *D.* Santa Rosa 'Purple Taffy'**: **1** (7.9%), **2** (0.9%), **3** (2.4%), **4** (32.5%),
210 **5**(18.1%), **6** (1.8%), A (0.1%) and B (1.0%).
211 **3.5.4. *D.* Sid Cywes 'Marlene'**: **1** (3.0%), **2** (2.1%), **3** (5.0%), **4** (30.6%), **5**
212 (37.1%), **6** (11.3%), A (1.2%) and B (6.0%).
213 **3.5.5. *D.* Unilangley 'Pink Tourmaline'**: **1** (5.9%), **2** (1.2%), **3** (5.7%), **4**
214 (53.8%), **5** (26.2%), **6** (0.3%), A (0.1%) and B (1.8%).
215
216 **4. Chemotaxonomic significance**
217 The occurrence of pelargonidin glycosides in the flowers of orchids were
218 previously established with thin layer and paper chromatography using
219 crude extracted pigments of the genera x*Brassotonia*, *Broughtonia*,
220 *Cattleopsis* and x*Cattleytonia* (Arditti, 1969). However, the identification
221 procedures of these orchid anthocyanins are considered to be rather out of

222 date and also lacking reliability due to absence of the data of the analysis by
223 HPLC, MS and so on (Griesbach, 1990). Therefore, the present results were
224 the exact report in which the distribution of pelargonidin glycosides and
225 acylated pelargonidin glycosides are confirmed in orchids.
226 Recently, anthocyanins have been used in chemotaxonomic studies of
227 Orchidaceae (Strack et al., 1986, 1989; Saito et al., 1994, 1995; Williams et
228 al., 2002; Fossen and Øvstedal, 2003; Tatsuzawa et al., 1994, 1996a,b, 1997, 1998,
229 2004, 2005, 2006, 2010b). These studies included the genera *Anacamptis*,
230 *Barlia*, *Bletilla*, *Cattleya*, *Cephalanthera*, *Cymbidium*, *Dactylorhiza*,
231 *Dendrobium*, *Dracula*, *Epipactis*, *Gymnadenia*, *Himantoglossum*, *Laelia*,
232 x*Laeliocattleya*, *Limodorum*, *Neottianthe*, *Nigritella*, *Ophrys*, *Orchis*,
233 *Phalaenopsis*, *Serapias*, *Sophronitis*, *Traunsteinera* and *Vanda*. Among these
234 genera, the 3,5-diglucoside pattern of anthocyanidin glycosides (including
235 3-glucoside and 3,7-diglucoside patterns) were detected from *Anacamptis*,
236 *Barlia*, *Cephalanthera*, *Dactylorhiza*, *Epipactis*, *Gymnadenia*,
237 *Himantoglossum*, *Limodorum*, *Neottianthe*, *Nigritella*, *Ophrys*, *Orchis*,
238 *Serapias* and *Traunsteinera* as their main anthocyanins (Strack et al., 1989).
239 From a standpoint of the phylogenetic classification of Orchidaceae, the
240 genera *Cephalanthera*, *Epipactis* and *Limodorum* belong to subfamily
241 Epidendroideae tribe Neottieae (Pridgeon et al., 2005), and the others belong
242 to subfamily Orchidoideae tribe Orchideae (Pridgeon et al., 2001). In this
243 study we found another member of the 3,5-diglucoside pattern of

anthocyanins for *Disa*. As the genus of *Disa* belongs to subfamily Orchidoideae tribe Diseae (Pridgeon et al., 2001), Diseae is the third orchid tribe other than Neottieae and Orchideae in which the 3,5-diglucoside pattern of anthocyanins were found.

To date, the distribution of 3,5-di-malonylglucosilated anthocyanins has been reported in the two families, Compositae and Labiatae (Takeda et al., 1986; Saito and Harborne, 1992). From a chemotaxonomical point of view, since *Disa* anthocyanins **5** and **6** were found to be 3,5-di-malonylglucosilated anthocyanins, the family of Orchidaceae, to which *Disa* belongs, should to be added to the above two families.

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February 7, 2011

Dr. M.S.J. Simmonds
Editor-in-Chief of Biochemical Systematics and Ecology

Dear Dr. M.S.J. Simmonds

Thank you very much for your e-mail dated February 4, 2011.
I am sending the revised manuscript entitled "Malonylated anthocyanidin 3,5-diglucosides in the flowers of the genus *Disa* (Orchidaceae)" by Fumi Tatsuzawa, Kazumitsu Miyoshi, Tomohisa Yukawa, Koich Shinoda, Kenjiro Toki, Norio Saito, Atsushi Shigihara, Toshio Honda.

We have revised all the points suggested by you as follows.

(1) According to the reviewer's comment, we mentioned Williams' work in the text and quoted as the reference.
(2) Although the NMR data for compounds **4** and **6** were not measured, unfortunately, the structure of **4** was unambiguously determined by direct comparison with the authentic specimen obtained by the hydrolysis of **5**. Therefore, we rewrote the sentence about the structures for pigments **4**-**6** in section 3.1.
(3) Section 3.2, p.5: We changed 'malonic acid was' to 'malonic acids were'.
(4) Section 3.2, p.5: We changed 'pigments 5' to 'pigment 5'.
(5) Section 3.2, p.6: According to the comment, the sentence 'Moreover, irradiations at H-1 of Glc A and B ……. were observed.' was deleted from section 3.2.
Moreover, the sentence 'Thus, malonic acids were attached to the OH-6 group of Glc A and B, respectively.' was moved after the sentence 'This result indicated that those two glucose units were acylated at the OH-6 groups with acids, respectively.'
(6) Section 3.2.1, p.7: According to the comment, we changed the coupling constants of H-5s, from 't, …..' to 'm'.
(7) Section 3.4, p.8: We changed 'acid was' to 'acids were'.
(8) Section 4, p.10: According to the comment, we changed 'detected' to 'established'.
(9) Section 4, p.11: We changed 'This is' to 'Therefore, the present results were'.
(10) Section 4: According to the comment, the sentence 'To date, the distribution of 3,5-di-malonylglucosilated anthocyanins has been reported in the two families, Compositae and Labiatae (Takeda et al., 1986; Saito and Harborne, 1992). From a

chemotaxonomical point of view, since *Disa* anthocyanins **5** and **6** were found to be 3,5-di-malonylglucosilated anthocyanins, the family of Orchidaceae, to which *Disa* belongs, should to be added to the above two families.' was added to the section 4.
(11) References: We changed 'Europian' to 'European'.

I hope that our manuscript will now be deemed worthy of publication in Biochemical Systematics and Ecology. Again, we thank you for your consideration of this manuscript.

With best regards,

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Figure 1

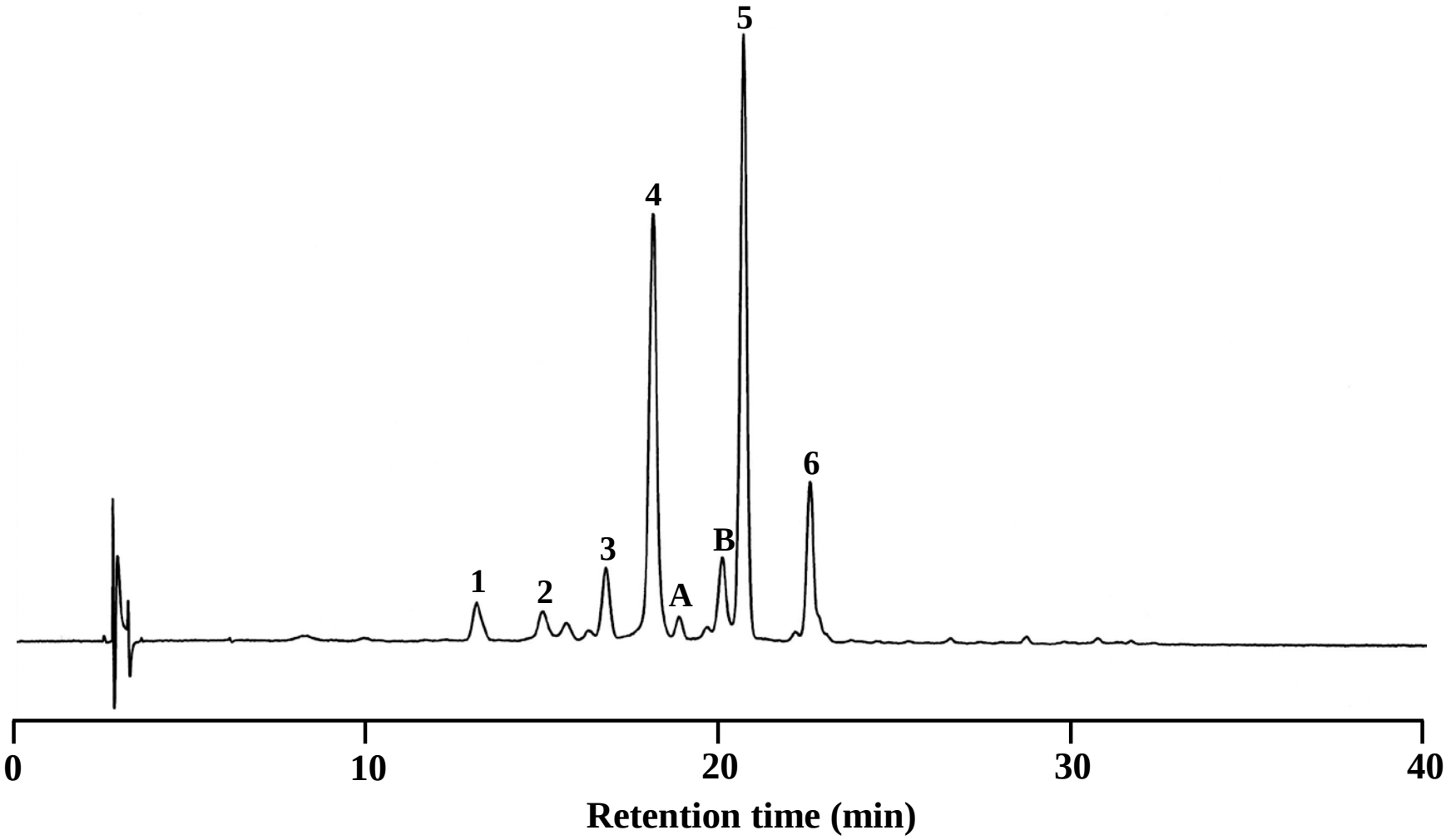


Figure 1. HPLC profile for anthocyanins (530 nm) in the red flower extract of *Disa* Sid Cywes 'Marlene'.

Pigments 1 - 6 are purified. Pigments A and B are not purified.

Figure 2

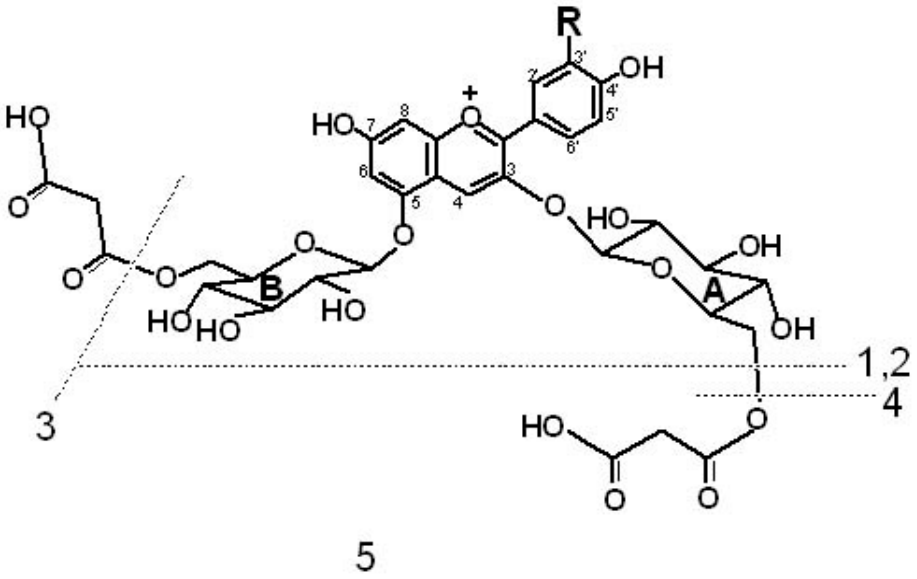


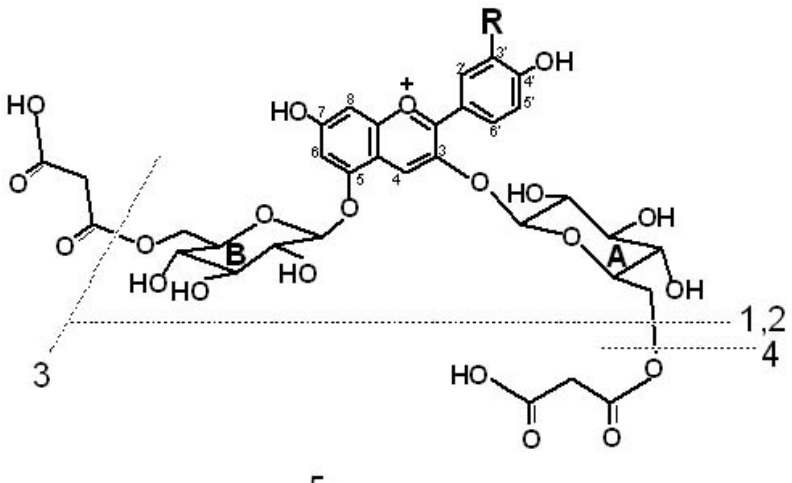
Figure 2. Anthocyanins from *Disa* cultivars.

1: R=OH, 2: R=H, 3: R=OH, 4: R=OH, 5: R=OH

Graphical Abstract

Malonylated anthocyanidin 3,5-diglucosides in the flowers of the genus *Disa* (Orchidaceae)

Fumi Tatsuzawa, Kazumitsu Miyoshi, Tomohisa Yukawa, Koich Shinoda, Kenjiro Toki, Norio Saito, Atsushi Shigihara, Toshio Honda



Anthocyanins from *Disa* cultivars.
1: R=OH, 2: R=H, 3: R=OH, 4: R=OH, 5: R=OH